THE OCCURRENCE OF PORPHYRIN IN THE PLANARIAN [1]

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During a study of electrophoretic properties of protein extracts of planarians, there was observed a substance characterized by a bright red fluorescence under ultra-violet light and a high electrophoretic mobility. The fluorescence suggested the substance could be a porphyrin or a related compound. Recent reviews in comparative animal biochemistry, although treating the occurrence of porphyrins, give no information as to the occurrence of porphyrin in planarians (Baldwin, 1939; Florkin, 1947; Prosser *et al.*, 1950).

An investigation of this substance with regard to some of its chemical properties identifying it as a porphyrin will be reported in this paper, and the possible biological significance will be discussed.

Materials

The biological material used was the planarian, *Dugesia dorotocephala;* the species is distinguished from related species by its large size (20–30 mm.) and dark pigment (Hyman, 1951). The planarians were collected in a pool on the Berkeley campus of the University of California. The water of the pool was covered by a dense growth of water fern among which the planarians were found. The temperature of the water varied between 14° and 16° C. during the summer when the animals were collected and studied. The animals were used for experiments on the next day or the same day as collected, unless stated otherwise.

Experimental Data

1. Preparation of extracts and preliminary results

The planarians were washed with distilled water and then homogenized in a Potter-type homogenizer. Distilled water was added to wash out the thick suspension. This water extract was centrifuged at low speed (about 500 rpm). The supernatant was discarded; the dark residue was resuspended in distilled water and centrifuged again. The washed residue was suspended in 0.01 *M* sodium carbonate at pH 11.0, in which solution almost all the residue dissolves. This was centrifuged and the pale brownish supernatant contained the substance fluorescing red under ultra-violet light. The original supernatant of the water extract was examined under ultra-violet; this possesses no red fluorescence but rather a pale gray fluorescence. No red fluorescence could be produced in the water-extracted supernatant by adding sodium carbonate up to the pH at which the dissolved residue

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fluoresces. This suggested that there was a red fluorescent substance bound to or adsorbed on the sedimentable granules that was not eluted with water but was extracted or eluted with aqueous sodium carbonate at pH 11.0.

The absorption spectrum of the residue dissolved in Na_2CO_3 was ascertained using a Beckman spectrophotometer. The spectrum showed a sharp absorption band near 400 mμ, as well as several weaker bands at the longer wave-lengths. The sharp absorption near 400 mμ is characteristic of the porphyrin ring and is found for all porphyrins regardless of their side chains. This band is termed the Soret band. The spectrum of the substance in Na_2CO_3 served only for its identification as a porphyrin. Using HCl as a solvent revealed the spectrum more clearly and permitted comparison with absorption maxima of porphyrins as reported in the literature; these maxima will be presented later in the paper.

2. Solubility study

The solubility of the porphyrin in various solvents was determined by suspending the water-insoluble residue in the solvent; the porphyrin was considered soluble if ultra-violet light [2] revealed red fluorescence in the solution. By this criterion, the porphyrin was found to be soluble in Na_2CO_3 (0.01 *M*), KOH (0.1 N), NH_4OH (10%), HCl (5% and 25%), and ethyl acetate (in acetic acid); it was insoluble in ethyl alcohol (95%), acetic acid (1%), chloroform, acetic-ether and ether. The fact that the porphyrin is insoluble in ether places it in the category of ether-insoluble porphyrins. This eliminates the possibility that the porphyrin in question is a protoporphyrin, mesoporphyrin, deuteroporphyrin or coproporphyrin, all of which are ether-soluble. The insolubility in ether and its solubility in ethyl acetate (in acetic acid) are characteristic of uroporphyrins, which are porphyrin octacarboxylic acids unextractable in ether or chloroform unless the carboxylic acid groups are esterified (Lemberg and Legge, 1949).

The methyl ester of the porphyrin was prepared by adding an acid solution of the porphyrin to cold methyl alcohol saturated with HCl, and allowing the solution to remain at 5° C. for several days. The porphyrin ester can then be extracted with chloroform. A hydrolysis of the ester can be achieved by adding 25% HCl to the methyl ester in chloroform and allowing the mixture to stand at 5° C. for several days. The red fluorescence of the porphyrin then appears in the top aqueous acid layer. This procedure was used for purification of the porphyrin for absorption spectrum studies.

3. Absorption spectrum study

The absorption spectrum of the planarian porphyrin in 5% HCl revealed maxima at I 600 mμ, II 555 mμ, III 495 mμ, and IV 406 mμ; order of intensity IV, III, II, I. When the porphyrin was dissolved in 25% HCl the maxima were I 598 mμ, II 553 mμ, III 496 mμ, and IV 409 mμ (see Fig. 1). The maxima reported by Grinstein, Schwartz and Watson (1945) for purified uroporphyrins in 25% HCl are: uroporphyrin (204° C. melting point) I 597 mμ, II 553 mμ, III 410 mμ; uroporphyrin (284° C. melting point) I 595 mμ, II 552 mμ, III 408 mμ.

[2] The source of ultra-violet radiation was a 6 watt GE360 Black Light lamp which produces long wave (3660 Å) ultra-violet. The lamp was supplied by Vogel Luminescence Corp.

The absorption spectrum of the methyl ester of the porphyrin in chloroform had maxima at I 626 mμ, II 582 mμ, III 575 mμ, IV 535 mμ, V 502 mμ, VI 408 mμ. The maxima reported by Grinstein *et al.* (1945) for the methyl ester of uroporphyrin in chloroform are I 626 mμ, II 581.5 mμ, III 570.5 mμ, IV 536 mμ, V 501.5 mμ, VI 408 mμ.

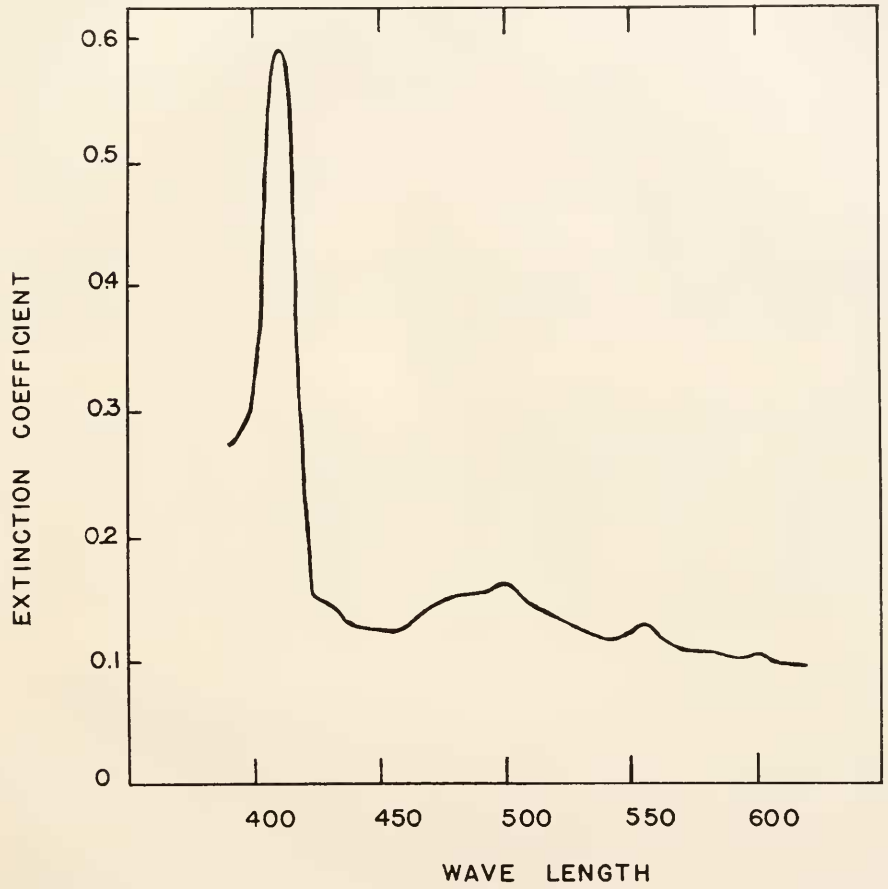

FIGURE 1. The absorption spectrum of the planarian porphyrin extracted in 25% HCl directly from water-insoluble granules is shown. The extinction coefficient ($\log \frac{I_o}{I}$) is plotted against the wave-length (in millimicrons).

After hydrolysis of the methyl ester with 25% HCl, the purified planarian porphyrin had maxima at I 596 mμ, II 553 mμ, (511 mμ) III 409 mμ; order of intensity III, II, I. After purification by esterification and hydrolysis, the absorption at 496 mμ is removed; this suggests that the absorption at 496 mμ was due to another substance, perhaps a related compound.

There is considerable similarity between the absorption spectrum of the por-

phyrin from planarians and the uroporphyrin isolated by Grinstein *et al.* (1945) from urine of patients with a congenital porphyria which is characterized by excretion of large quantities of uroporphyrin.

4. Paper electrophoresis

The mobility of the porphyrin was observed with paper electrophoresis using a somewhat modified technique of Kunkel and Tiselius (1951). Whatman 3MM filter paper was held between glass plates and dipped at the ends into electrode vessels containing Na_2CO_3 (0.01 *M*), $\mu = 0.03$, pH 11.0. The porphyrin solution was placed on the paper at the cathode end 17 cms. from the cathode and 40 cms. from the anode. The run was made at room temperature using 200 volts and 3–5 milliamperes. Serum albumin stained with brom phenol blue was applied parallel to the porphyrin for comparison of relative mobilities. Both the stained albumin and the red fluorescent spot of the porphyrin moved as discrete areas towards the anode. The location of the anterior edge of the two moving areas was noted at intervals. The distance moved for porphyrin and albumin, respectively, was 5.7 cms. and 3.8 cms. after one hour; 9.7 cms. and 6.0 cms. after two hours. The ratios of their relative mobilities were 1.45 at one hour and 1.6 at two hours.

To locate protein at the completion of the run, the paper was immersed in 10% $HgCl_2$ in 95% ethyl alcohol, stained in brom phenol blue (0.1% in 95% ethyl alcohol) for one hour, destained in 1% acetic acid. No protein stain was observed at the final location of the red fluorescence, although the protein staining revealed a protein of low mobility not associated with the red fluorescence under these conditions of electrophoresis. The protein may have dissociated itself from the porphyrin during the electrophoresis or may be a separate protein found in the residue and extracted by the same alkaline solvent as the porphyrin.

5. Paper chromatography

The identification of the planarian porphyrin was checked by paper partition chromatography using a lutidine-water system described by Nicholas and Rimington (1951). There is a linear relationship between R_F values and the number of carboxyl groups in the porphyrins and this can be used to identify the porphyrin.

The porphyrin was extracted in 2 N NH_4OH or 1% HCl and applied in the solvents to pieces (12 × 15 cms.) of Whatman No. 1 filter paper along a base line 2 cm. from the lower edge. The chromatographs were run in the dark at 21° C. in a lutidine-water system containing 40% water by volume. The position of the spots after development was determined by their fluorescence in ultra-violet light.

The porphyrins run were the planarian porphyrin assumed to be a uroporphyrin and a known sample of hematoporphyrin. R_F values were calculated and compared. Under these experimental conditions, the R_F value of the hematoporphyrin was between 0.7 and 0.8; the R_F value of the planarian porphyrin was between 0.01 and 0.1. The R_F values reported by Nicholas and Rimington (1951) for two-carboxyl porphyrins (such as hematoporphyrin) were about 0.8 and for eight-carboxyl porphyrins (uroporphyrin) were about 0.1 under experimental conditions similar to

those used in these experiments. This suggests that the planarian porphyrin contains eight carboxyl groups and may be uroporphyrin.[3]

6. Content of porphyrin in planarians

To determine whether porphyrin content varied from one planarian to another, different numbers of animals of approximately the same size were collected and the relative amount of porphyrin determined. The extinction coefficient ($\log \frac{I_o}{I}$) at the maximum absorption, 408 mμ, was used as the measure of porphyrin content. Since planarians vary in size, it is necessary to relate the porphyrin to some unit of planarian size or weight. Wet weight was chosen as the most convenient measure. Since the porphyrin is insoluble in ethyl alcohol, this can be used as a means of killing or fixing the animals and thus facilitating wet weight determinations. Before extraction of the porphyrin, a known number of planarians was placed in a tared watch glass, the animals were immediately killed with 95% ethyl alcohol, and filter paper was applied to remove excess moisture before weighing. After weighing, the planarians were homogenized in water, centrifuged, the residue extracted

Table I

Number of planarians	Extinction at 408 mμ	Total wet weight (mg.)	Ratio (E/weight)
2	0.081	13.7	0.0059
4	0.256	41.6	0.0061
6	0.335	51.7	0.0064
8	0.412	60.9	0.0067
10	0.482	71.2	0.0067

in a constant volume (3 ml.) of 25% HCl, and the extinction coefficient determined. The porphyrin coefficient was stated in arbitrary units as the extinction at 408 mμ per total wet weight. The data are presented in Table I. Under similar physiological conditions, the unit of porphyrin per planarian of a given wet weight may be a constant.

Preliminary experiments indicate that the porphyrin is also present in animals which had been starved in the laboratory for one, two and four weeks. Porphyrin is present in regenerating and regenerated animals. The porphyrin is not confined to any one region of the animal; it is distributed throughout the animal although the distribution may not be uniform along the length of the animal.

7. Visual demonstration of porphyrin

A visual demonstration of porphyrin within the planarian can be made by exposing animals to ultra-violet light after they have been immersed in 95% ethyl

[3] A sample of the planarian porphyrin was analyzed recently in the laboratory of Dr. Samuel Schwartz of the Department of Medicine at the University of Minnesota. More refined techniques of chromatography on calcium carbonate revealed two red fluorescent zones. Paper chromatography showed the presence of coproporphyrin and a porphyrin which behaves like uroporphyrin. The author wishes to thank Dr. Samuel Schwartz, as well as Dr. Marie Berg and Mr. Micheal Keprios for their analysis of this material.

alcohol. One can then observe the whole planarian emitting a red fluorescence. No definite fluorescence could be detected in the living animal, although the water-insoluble sedimentable granules do show some fluorescence without further chemical treatment. The alcohol removes from the animal a substance or substances which may obscure the red fluorescence. This substance is yellow in solution, has a yellow fluorescence, and is soluble in organic solvents such as ethyl alcohol, methyl alcohol, acetone, chloroform and pyridine. Any of these organic solvents can remove the yellow substance from the whole animal and allow the red fluorescence of the porphyrin to be observed. This yellow substance is probably a lipochrome or carotenoid, and requires further investigation.

Discussion

Uroporphyrins were first described by Fischer in 1915 and since then much work has been done to characterize and describe this substance (see review by Lemberg and Legge, 1949). The main source of uroporphyrin has been the urine of patients having congenital porphyria (Waldenström *et al.*, 1935; Grinstein *et al.*, 1945). In the case of porphyria the occurrence of uroporphyrin is considered to be a metabolic error. However, in the case of the planarian, this porphyrin seems to be found in all individuals and may occur as a physiological porphyria, as Turner (1937) described the case in the fox-squirrel where uroporphyrin occurs normally in the bones. Uroporphyrin is found normally in small amounts in the zones of calcification of foetal bones and it is found in larger amounts in the bones and teeth of congenital porphyria cases in man and other mammals (Rimington, 1955). Uroporphyrin occurs in the *Pteria* mussel shell (Fischer and Haarer, 1932; Nicholas and Comfort, 1949) and occurs as a copper complex in the flight feathers of the African turacos (Fischer and Hilger, 1924). In these cases the uroporphyrin seems to be stored in metabolically rather inert tissues, except perhaps for foetal bones. This suggests that the uroporphyrin may be an end-product of chemical events, perhaps of calcification in some cases. Uroporphyrin has been postulated to be in the biosynthetic pathway leading to the formation of haem, a porphyrin containing substance vital to higher organisms, but recent studies suggest that uroporphyrin may be a side reaction and not involved in the biosynthetic pathway to haem (see review of Rimington, 1955). Thus uroporphyrin may be an end product whenever it occurs, either in pathological urine, bones, teeth, feathers, shells or in planarians. However, in the case of the planarian, the porphyrin is distributed rather generally and is not stored in inert tissue. This raises the question whether there may not be some biological significance for the planarian porphyrin.

Loeb (1893) and Hesse (1897) had independently observed that the movements of planarians were influenced by light. Parker and Burnett (1900) extended these observations and measured the speed of movement of planarians away from light; they found that animals with and without eyes responded so as to move away from light but the eyeless ones at a somewhat slower rate than those with eyes. It has also been shown that planarians perceive ultra-violet radiations and produce a negative reaction; the behavior is the same for normal and eyeless specimens (Werner, 1926). Short exposures to ultra-violet and to direct sunlight are fatal to planarians (Merker and Gilbert, 1932).

The question is whether this sensitivity and response to ultra-violet may wholly or in part perhaps be due to the presence of porphyrins which are strongly photoactive and efficient in absorbing light around 4000 Å. This response may be related to the photosensitizing action of porphyrins discovered in 1909 by Hausmann (as reported by Lemberg and Legge, 1949). He found that experimental animals and humans injected with porphyrin solutions and then exposed to sunlight or ultra-violet light, showed a skin sensitization. Also it is known that human individuals suffering from congenital porphyria show a marked light sensitization of the skin (Lemberg and Legge, 1949). In planarians the light sensitization may have a normal function to produce the avoidance of light.

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Summary

1. A porphyrin was extracted from the water-insoluble residue of homogenates of the planarian, *Dugesia dorotocephala*. The porphyrin extract is characterized by a red fluorescence under ultra-violet light.

2. The solubility properties of the porphyrin, the absorption spectra of the porphyrin and its methyl ester, and paper chromatographic behavior show a similarity to those described for uroporphyrin.

3. The amount of porphyrin seems to be a constant per planarian of a given wet weight, when the animals are under the same physiological condition. The porphyrin is also found in starved and in regenerating animals.

4. The whole planarian emits a red fluorescence under ultra-violet light if the animal is first immersed in one of several organic solvents which remove a lipochrome or carotenoid substance.

5. The light sensitivity which has often been described for these animals may to some degree be due to the presence of the porphyrin.

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